

Antigen-independent selection of intracellular stable antibody frameworks

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Accepted 8 April 2004

Available online 8 July 2004

Abstract

The intracellular expression of highly specific antibody fragments (“intrabodies”) in eukaryotes has a great potential in functional genomics and therapeutics. However, since the intracellular reducing environment prevents formation of the conserved intrachain disulfide bonds, most antibodies do not fold properly and are therefore inactive inside cells. The few antibodies that have been found to function in an intracellular environment and that have been characterized for their biophysical properties have generally shown a high degree of stability and solubility. Thus, for intracellular expression and application, very stable antibody frameworks are needed that can correctly fold even in the absence of disulfide bonds and that do not aggregate. Here, we present and discuss a novel method, named “Quality Control,” which allows selection of stable and soluble antibody frameworks *in vivo* without the requirement or knowledge of antigens. This system is based on the expression of single-chain antibody fragments (scFvs) fused to a selectable marker that can control gene expression and cell growth. The activity of such a selectable marker fused to various scFvs that have been biophysically characterized correlated with the solubility and stability of the scFv moieties. This antigen-independent intrabody selection system was applied to screen scFv libraries for identifying stable and soluble frameworks, which subsequently served as acceptor backbones to construct intrabody libraries by randomization of hypervariable loops.

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Keywords: Stable antibodies; Antibody framework; Intracellular antibodies; Intrabodies; Antibody selection; Antibody expression; Protein knock-out; Domain knockout; Target validation; Gene therapy

1. Introduction

The ability of antibodies to bind almost any molecule with a high degree of specificity and affinity has been exploited to turn these proteins into powerful therapeutic and diagnostic tools. Advances in recombinant DNA technology have facilitated the manipulation and expression of antibodies in a wide variety of hosts, and several forms of antibodies have been constructed to obtain derivatives that carry the antigen-binding site in a smaller assembly. One of the minimal forms still retaining full binding activity is the single-chain Fv fragment (scFv), in which the variable regions of the heavy and the light chains are connected by a flexible peptide linker,

allowing the expression of the protein from a single cDNA sequence [1,2]. In addition to conventional extracellular applications, the scFv antibody form can in principle be readily expressed also in the cytoplasm of eukaryotic cells and directed to any compartment to target intracellular proteins and thus evoke specific biological effects. Thus, intracellular scFvs, also known as “intrabodies,” have a great potential in functional genomics by blocking or modulating the activity of proteins and protein domains, thereby contributing to the understanding of their functions [3]. In the long run, intrabodies might even have therapeutic applications, possibly in gene therapy settings [4].

In spite of this exciting perspective and a few examples of successful applications [5–9], the cytoplasmic expression of scFvs is generally limited by their instability and insolubility. The reducing environment of the cytoplasm prevents the formation of the conserved

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intrachain disulfide bridges, thus rendering a high percentage of scFvs unstable and, as a consequence, non-functional inside the cell [10–12]. Highly stable and soluble antibody frameworks, which can fold even in the absence of the disulfide bonds and do not undergo aggregation, are therefore required for the application of intrabodies as potential modulators of protein function *in vivo*. Since only a small fraction of a typical scFv library will generally have sufficient stability for intracellular application [13], one approach to obtain a much higher number of functional intrabodies can be provided by constructing randomized hypervariable loop libraries on scFv frameworks that have been pre-selected for high stability and solubility in an intracellular environment.

The method described here, called “Quality Control,” allows rapid selection of stable and soluble antibody frameworks in yeast cells independently of their antigen-binding specificity [14]. In our system, the activity of a selectable marker protein fused to an scFv strictly correlates with the stability and solubility of the scFv moiety. Since the selectable marker used in our assay can control cell growth by activating transcription of a conditionally essential gene, yeast colony formation on a plate, or growth in liquid culture, depends on the fusion with a stable and soluble scFv antibody fragment. The “Quality Control” system for antibody frameworks was first tested by fusing the selectable marker protein to various anti-GCN4 scFvs that have been previously characterized *in vitro* for stability and solubility, as well as *in vivo* for their performance as antigen-specific antibodies [12]. Our results show that only stable and soluble scFv moieties permit activation of the specific reporter genes. Subsequently, the “Quality Control” system was efficiently applied to screen scFv libraries for isolating “super-stable” frameworks suitable for intracellular as well as extracellular applications.

2. Description of methods

2.1. Principle of the method

The method presented here to identify intracellular stable and soluble scFv antibody frameworks is based on the hypothesis that stability and solubility (and therefore the activity) of a fusion protein composed of a constant, selectable marker fused to an scFv is determined by the quality of the scFv moiety. To test this hypothesis, and to prove the principle of the method, we fused a number of well-characterized scFvs to two types of marker proteins, both of which, when expressed in an active form, can specifically activate expression of reporter genes. Fig. 1 depicts the principle of our method as tested with the marker protein composed of a peptide linked to the transcriptional activation domain (AD) of Gal4. The peptide was derived from the mutant form of the yeast protein

Gal11 called Gal11P. It has been previously shown that only the mutant Gal11P peptide, but not the wild-type sequence, specifically interacts with the dimerization region of the DNA-binding Gal4 fragment comprising the first 100 amino acids [15]. The activation domain of Gal4 is known to activate transcription when tethered to DNA via protein–protein interactions [16]. According to the hypothesis outlined above, a fusion protein bearing a soluble and stable scFv should be tethered to DNA via the Gal4(1–100)–Gal11P interaction and, thus, activate transcription of the reporter genes, whereas an unstable and/or insoluble scFv should render the entire fusion protein inactive (Fig. 1). The divergently oriented *lacZ* and *HIS3* reporter genes allow quantification of the level of gene activation and positive growth selection on selective media, respectively. The possibility to select for activation-dependent cell growth is particularly useful for screening antibody framework libraries fused to the marker protein in order to identify those that are stable and soluble in an intracellular environment (see below).

2.2. Antibody framework-dependent gene activation

To develop and validate our system, several defined scFv fragments displaying different *in vitro* stabilities and solubilities were fused to two types of selectable marker proteins for intracellular expression [14]. On one hand, the Gal11P–Gal4AD marker protein is depicted in Fig. 1, and, on the other hand, the transcriptional activation domain of Gal4 directly fused to the DNA-binding LexA protein (LexA–Gal4AD) [17]. The Gal4 activation domain (amino acids 768–881) was amplified by PCR using pGAD424 (Clontech) as template with primers including the SV40 T-antigen nuclear localization signal N-terminal to the Gal4-AD. The DNA-fragments encoding amino acids 263–352 of Gal11P were amplified by PCR and cloned in-frame, N-terminal to the SV40-NLS-Gal4-AD-construct. For the direct DNA-binding fusion construct, the LexA full-length protein (amino acids 1–202) and the Gal4 activation domain (amino acids 768–881) were fused together. While the Gal11P–Gal4AD marker protein is relatively short, flexible, and most likely a monomer, the LexA derivative is larger, globular, and capable of forming dimers [18]. It was therefore assumed that the stability and function of the latter would be less readily affected by the qualities of the fused scFv moieties. The test scFvs, all of which bind the so-called leucine zipper of the yeast transcription factor Gcn4, have been extensively characterized for their biophysical properties, as well as for their *in vivo* performance as inhibitors of the Gcn4 transcriptional activity in yeast [12]. The original anti-GCN4 wild-type (anti-GCN4wt) scFv was obtained by ribosome display from a library constructed from an immunized mouse [19]. A destabilizing point mutation introduced in the heavy chain (anti-GCN4H-R66K) was shown to decrease both

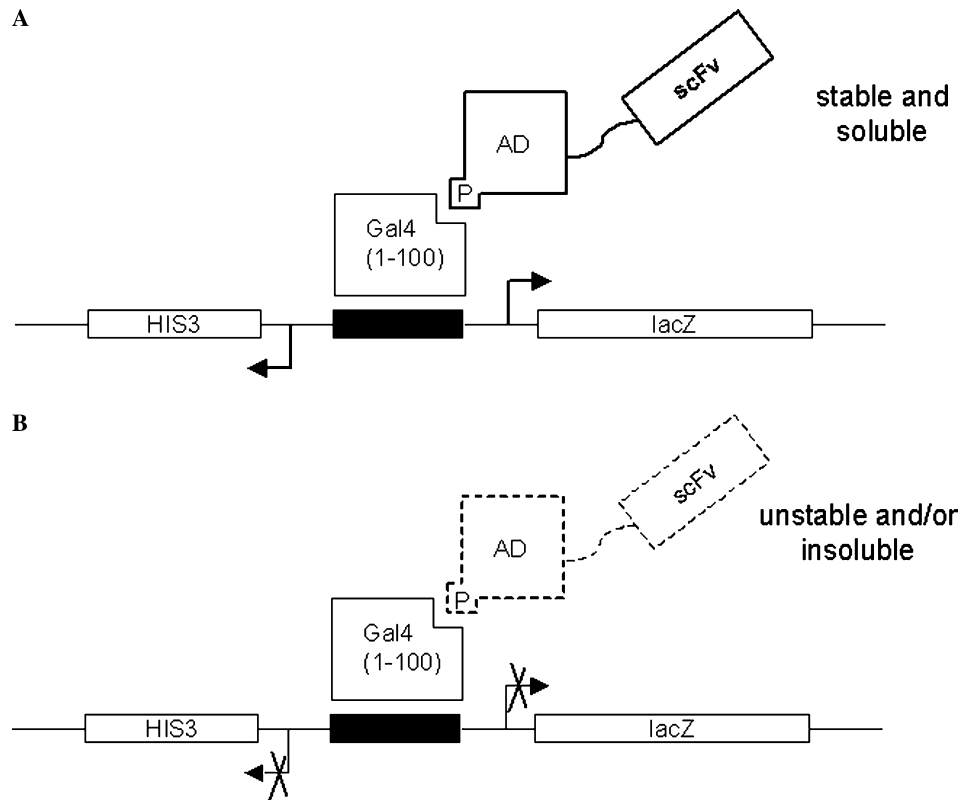


Fig. 1. Outline of a gene expression assay applied in the “Quality Control” system for the antigen-independent selection of intracellular stable antibody frameworks. Single-chain Fv antibody fragments (scFv) are fused to a selectable marker protein comprising a transcriptional activation domain (AD) and a peptide derived from Gal11P (P), which can mediate the specific interaction with the DNA-bound Gal4 (1–100) fragment. The basic concept of the “Quality Control” system is that the activity of the entire fusion protein carrying the selectable marker is dependent on the stability and solubility of the scFv moiety. (A) A fusion protein carrying a stable and soluble scFv is tethered to DNA via the Gal4(1–100)–Gal11P interaction where it activates transcription of the divergently oriented *lacZ* and *HIS3* reporter genes (bent arrows). (B) Unstable and/or insoluble scFv moieties lead to inactivation of the entire fusion protein (dashed lines), thus impeding activation of transcription of the reporter genes (crossed arrows).

in vitro stability and in vivo performance. Similar results were obtained with a cysteine-free anti-GCN4 fragment (anti-GCN4SS^{−−}), in which both intradomain disulfides were replaced by Val–Ala pairs. In contrast, a framework-engineered stabilized version, obtained by grafting the CDR loops in a λ -like fashion on a stable framework termed λ -graft [20], showed much higher in vitro stability and in vivo performance as inhibitor of Gcn4 activity [12]. Another framework-engineered version, obtained by grafting the CDR loops in a κ -like fashion on the same stable framework (κ -graft), was shown to be highly stable but to display a much lower Gcn4-binding affinity [12]. More recently, a so-called Ω -graft, which differs from the λ -graft by only three amino acids in the framework sequence, showed 30–40% higher activity than the λ -graft in an in vivo interaction assay [21].

The influence of the various scFvs described above on the activity of the fusion protein markers in our “Quality Control” system was tested as follows. The yeast strain YDE172 containing a *lacZ* reporter gene under the control of four Gal4-binding sites [14] was transformed according to a standard lithium acetate transformation protocol with the indicated scFvs fused to the Gal11P–

Gal4AD marker protein along with another plasmid expressing the DNA-binding Gal4 (1–100) domain. Expression of the scFv fusion proteins is under the control of the *ACT1* promoter from a 2 μ m plasmid carrying the *TRP1* nutritional marker gene. An *ARS/CEN* plasmid with the *LEU2* marker gene was used to express the DNA-binding Gal4 (1–100) fragment under the control of the *ACT1* promoter. A different yeast strain (YDE173) carrying the *lacZ* reporter gene under the control of six LexA-binding sites was transformed with the same scFv variants fused to the artificial transcriptional activator LexA–Gal4AD [14]. Expression of the LexA fusion proteins is under the control of the ADH promoter from an *ARS/CEN* plasmid harboring a *LEU2* nutritional marker gene. Following transformation, the cells were plated on appropriate amino acid drop-out plates [22]. Yeast cells expressing these different proteins were grown in culture and subjected to a β -galactosidase assay to quantify expression of the *lacZ* reporter gene. Duplicates of 2 ml overnight-cultures were inoculated in drop-out medium (–Trp) from streaks containing several colonies and grown at 30 °C. Cultures were diluted in 1 ml respective drop-out medium to an

optical density at 600 nm (OD_{600}) of 0.7. They were grown at 30 °C for 2 h. For the assay 100 μ l cell culture was taken, mixed with 900 μ l buffer, 45 μ l chloroform, and 30 μ l of 0.1% SDS, mixed, and incubated at room temperature for 5 min. The color development was initiated by the addition of 0.2 ml ONPG (4 mg/ml) and stopped with 0.5 ml Na_2CO_3 (1 M). The activity was calculated by taking into account the OD_{600} of the assay culture, as well as the incubation time of the color development and the culture volume used. Cells expressing the full-length transcriptional activator Gal4 or the hybrid activator LexA–Gal11 [15] were used as positive controls. Cells transformed with an empty plasmid served as negative control. Fig. 2A shows that the Gal11P fusion proteins bearing the stable scFv moieties called λ -graft and κ -graft [12] were able to strongly activate *lacZ* expression, while those harboring the unstable scFvs (anti-GCN4wt, anti-GCN4H-R66K, anti-GCN4SS⁻ [12]) were unable to stimulate *lacZ* expression. The so-called Ω -graft displayed an approximately

40% better in vivo performance than the λ -graft, which is similar to the performance observed in antigen-dependent in vivo interaction experiments [21]. Very similar results were obtained by performing β -galactosidase assays with the yeast strain YDE173 expressing the LexA–Gal4AD–scFv fusion proteins (Fig. 2A, striped bars), thus indicating that the scFv moieties had a similar and dominant effect on the activity of both types of selectable marker proteins.

In summary, these results, taken together with the biochemical characterization of the various scFvs [12], show a correlation between the levels of reporter gene activation by the fusion proteins and the in vitro stability and solubility of the single-chain moiety.

2.3. Growth selection of stable and soluble scFv antibody frameworks

Both the YDE172 and the YDE173 yeast strains also contain *HIS3* reporter genes that are under the control

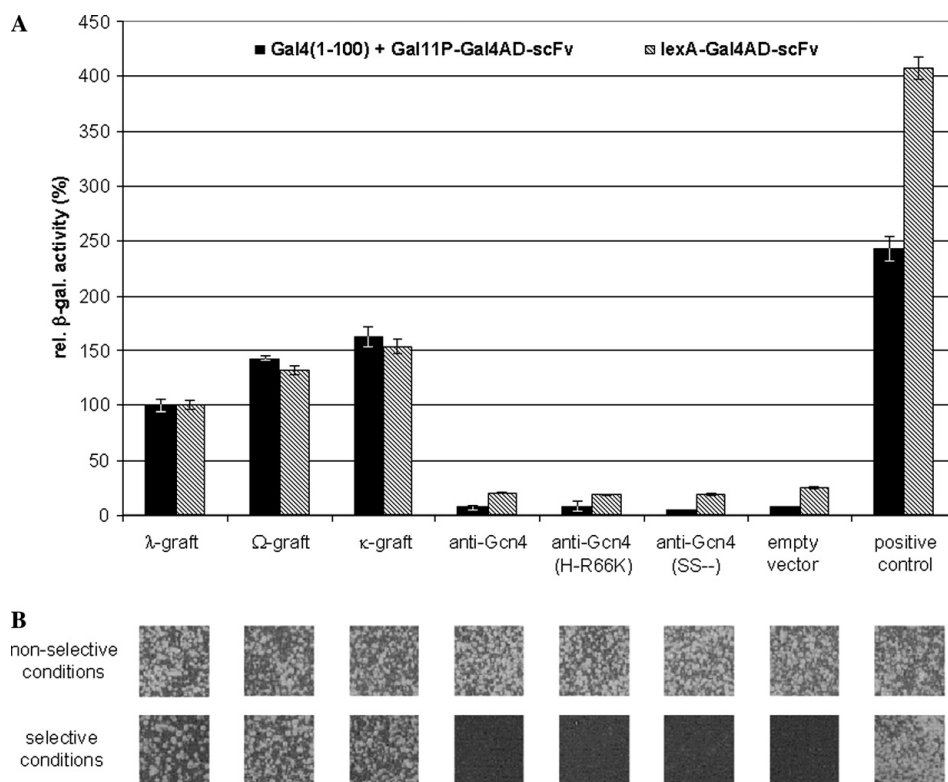


Fig. 2. The degree of reporter gene activation correlates with the stability and solubility of the scFv moiety. (A) β -Galactosidase assay to measure activation of the *lacZ* reporter gene. The reporter yeast strain carrying the *lacZ* gene under the control of four Gal4-binding sites was transformed with plasmids expressing the respective Gal11P–Gal4AD (activation domain) scFv fusion proteins together with the DNA-binding Gal4(1–100) fragment. A second reporter yeast strain harboring the *lacZ* gene under the control of six LexA-binding sites was transformed with the same scFv variants fused to the strong transcriptional activator LexA–Gal4AD. Gal4 full-length and LexA–Gal11 were used as positive controls. Cells transformed with an empty vector served as negative controls. The soluble and highly stable Ω -, λ -, and κ -graft scFv variants enabled the respective fusion proteins to strongly activate the *lacZ* reporter gene, while the less stable scFv variants (anti-GCN4wt, anti-GCN4H-R66K, and anti-GCN4SS⁻) did not allow significant reporter gene activation. Relative β -galactosidase activity (rel. β -gal activity) elicited by the λ -graft fusion proteins was arbitrarily set to 100%. (B) Growth on selective plates containing 3-aminotriazole (3-AT) correlates with the gene activation potential of the tested scFv fusion proteins. The reporter yeast cells transformed with plasmids expressing the scFv variants in the context of the Gal11P–Gal4AD fusion were spread on selective plates lacking histidine and supplemented with 0 mM (non-selective conditions) or 20 mM 3-AT (selective conditions). Cell growth was monitored after 72 h incubation at 30 °C.

of four Gal4-binding sites or six LexA-binding sites, respectively (see Fig. 1 for an outline). Activation of these reporter genes allows growth selection on plates lacking histidine and containing 3-aminotriazole (3-AT), which is a competitive inhibitor of the *HIS3* gene product. The same yeast cells that were subjected to the liquid β -galactosidase assay (Fig. 2A) were spread on selective plates lacking histidine and containing different concentrations of 3-AT, as well as on non-selective control plates (–Trp/–Leu only). Plates were incubated at 30 °C and colony formation was monitored. Under non-selective conditions, no difference in the growth rate was observed between yeast cells harboring the various scFv fusion variants, indicating that none of the constructs was toxic to the cell. Under selective conditions, a 20 mM 3-AT concentration was already sufficient to suppress growth of yeast cells carrying an empty vector or expressing a fusion protein with unstable and insoluble scFv moieties (Fig. 2B). Even after an observation period of four days, yeast cells expressing unstable fusion variants were unable to grow in the presence of 3-AT concentrations ranging from 10 to 60 mM (data not shown). In contrast, stable and soluble scFv moieties such as the λ -graft, the κ -graft, and the Ω -graft endowed the respective fusion proteins with the ability to stimulate growth of the yeast cells under these selective conditions (Fig. 2B). These results indicate that this growth selection procedure allows distinguishing intracellular stable scFvs from unstable ones and permits efficient screens of scFv libraries to isolate “super-stable” frameworks suitable for intracellular applications.

2.4. Construction of scFv-fusion libraries for intrabody selection in yeast

To screen scFv libraries in the “Quality Control” system for identifying stable and soluble frameworks, either one of the two types of fusion constructs described above was used. On the one hand, a fusion of the scFv-library to the Gal4 activation domain linked to the Gal11P-peptide, and on the other hand, a direct fusion of the scFv-library to the DNA-binding and activating LexA–Gal4AD. A defined scFv can be fused to the N- or the C-terminus in both cases without showing a significant difference in the activity of the marker protein (data not shown). For screening purposes, however, the scFv-library is best fused to the N-terminus to avoid getting large numbers of false positives. Otherwise, in the case of C-terminal fusions, all mutations introduced by PCR resulting in a stop codon would lead to the expression of truncated versions of the fusion construct. Such truncated versions would then contain essentially the Gal4–Gal11P or LexA–Gal4AD fusions, which are by themselves very strong activators of transcription.

The plasmids for expression of the scFv library-fusion constructs for screening in yeast were derived from

pESBA-ACT. This vector contains the yeast *TRP1* gene for transformation selection in *Saccharomyces cerevisiae* and the 2 μ m origin of replication to ensure high copy numbers. The fusion protein is inserted between a constitutive actin (*ACT1*) gene promoter for strong expression and the *GAL11* transcriptional termination sequence. For handling in bacterial systems, the vector also has a bacterial origin of replication and the amp resistance gene.

Human DNA sequences encoding the antibody heavy and light chain variable domains were amplified from human spleen-cell cDNA using a large primer set as described previously [23–25] and cloned via overlap extension PCR in the orientation V_L-linker-V_H, where the linker has the sequence (GGGS)₄. The resulting scFv library was inserted in-frame into the yeast vector via SfiI-sites, N-terminal to the two types of transcription-activating constructs described above.

2.5. Antigen-independent screening of scFv libraries in yeast to select intrabody frameworks

The various steps of the screening protocol for the selection of intrabody frameworks are schematically outlined in Fig. 3. In this procedure, the yeast strains containing the reporter systems described above were transformed with the appropriate scFv-library fusion constructs by following a standard lithium acetate transformation protocol [22]. In the case of the scFv–Gal11P–Gal4AD fusion, cells were co-transformed with the plasmid expressing Gal4 (1–100) to permit reporter gene activation. Following transformation, cells were plated on drop-out plates that also selected for expression of the *HIS3* reporter gene. Selective conditions were –Trp/–Leu/–His plates containing varying concentrations of 3-aminotriazole (3-AT) up to 80 mM. An aliquot of the transformed yeast cells was plated on drop-out plates that were not selective for *HIS3* expression to allow estimation of the number of screened clones. Colonies were picked from the selective plates after 3 days incubation at 30 °C, re-streaked on selective drop-out plates, and subsequently tested for *lacZ* expression in a filter assay in the presence of the β -galactosidase substrate X-Gal according to standard protocol [22]. From positive yeast cells, which showed *lacZ* reporter gene activation, library plasmids were isolated. Fresh yeast cells were transformed with these plasmids and the activity of β -galactosidase was evaluated quantitatively in a liquid assay in extracts of these newly transformed cells, as described in the previous section.

Only about 0.1% of all the colonies screened using either of the two library-fusion constructs was able to grow under the selective conditions (Table 1). Such small number of colonies growing under selective conditions demonstrates a high degree of selectivity of the “Quality Control” system towards stability and solubility of the

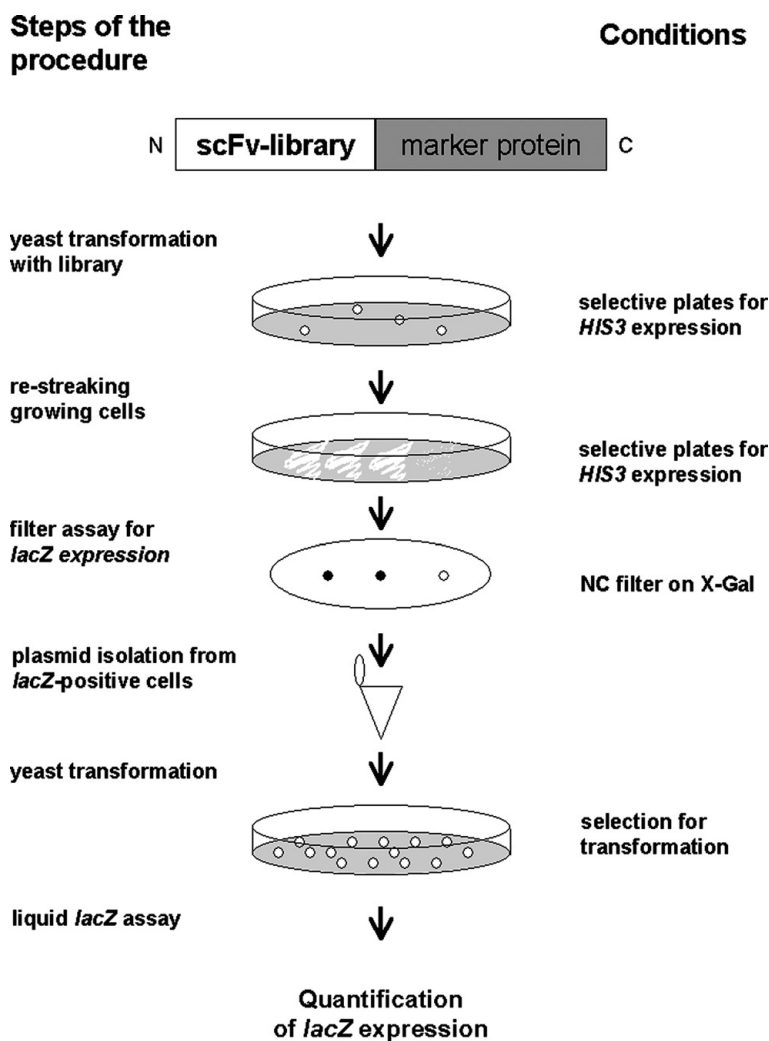


Fig. 3. Schematic representation of the “Quality Control” screening procedure. On the left side, the sequence of the experimental steps is given, while the right side lists the necessary selective conditions at each step during the screening procedure. All methods are either carried out according to standard procedures, or as described in the sections presenting the experiments with the defined anti-GCN4 scFvs. Briefly, the scFv-library fused N-terminally to either of the two described marker proteins is transformed into the corresponding yeast strain containing a selectable reporter gene. Depending on the strain used, selective conditions are chosen between 0 mM (only –Trp, –Leu, and –His) and 80 mM 3-AT, to minimize background growth. Growth-positive clones are picked after 3 days incubation at 30 °C and tested by re-streaking the cells under the same selective conditions and, subsequently, by measuring expression of the independent *lacZ* reporter gene on a filter assay using nitrocellulose (NC) filters for colony transfer on plates containing the β -galactosidase substrate X-Gal. Plasmid isolation from yeast is performed for confirmed positive clones and isolated library members are finally tested for *lacZ* activation in the same yeast strain used for selection, without, however, exposing it to selective conditions. This is to avoid growth selection pressure on the yeast cells and thus the occurrence of false positives. Those freshly transformed clones are then processed in a liquid *lacZ* assay to quantify the strength of reporter gene expression and thus rank the performance of the selected molecule.

Table 1
Selectivity of “Quality Control” screens for intrabody frameworks

Total clones screened in yeast	1×10^7
Percentage of screened clones allowing cell growth under selective conditions	~0.1%
Percentage of growth-selected clones that show scFv-dependent <i>lacZ</i> expression	~60%

expressed scFv fusion constructs. In any case, we considered only those positive clones for which activation of the independent *lacZ* reporter gene could be confirmed upon isolation of the library plasmid and transformation

of freshly prepared yeast cells, which had not been subjected to selective pressure (Table 1). The occurrence of false-positive clones has been observed at different steps of the procedure. On the one hand, those colonies that do not re-grow after picking them from the screening plates: on the other hand, those that can be confirmed for growth, but are not able to activate transcription of the *lacZ* reporter gene. In the latter case, failure to activate transcription of the *lacZ* gene can either be observed already in the context of the yeast cells subjected to growth selection conditions or just after reintroduction of the isolated plasmid into the original yeast strain.

Using the Gal11P-interaction system for screening, most of the false-positive clones were already identified at the step of re-growth on selective medium following picking from the screening plates. In the case of the direct fusion to the DNA-binding and activating LexA–Gal4AD, however, most colonies that grew on selective plates were also initially positive for activation of the independent reporter gene *lacZ*, and false-positive clones were only discovered after plasmid rescue and the second *lacZ* assay. The reason for this observed difference between the two types of hybrid proteins is unknown.

Analysis of more than 50 framework sequences that were selected in the “Quality Control” system showed that they belong to subfamilies of variable domains with a track record of high stability and good folding properties in vitro [26]. In agreement with this sequence analysis, the biophysical characterization of five of the selected scFv frameworks in vitro confirmed their high degree of stability and solubility under various conditions. Moreover, production of these antibody fragments from bacteria gave significantly higher yields than those obtained with average antibodies, i.e., over 100 mg

protein per liter culture. A detailed description of the sequence analysis and the results of the biophysical characterization of selected frameworks will be presented elsewhere (manuscript in preparation).

2.6. Similar intrabody framework behavior in yeast and in mammalian cells

To determine whether these characterized scFvs have the same properties when expressed in mammalian cells, we modified our “Quality Control” system for expression in tissue culture cells. The scFv fragments tested in yeast (see above) were fused at the C-terminus of a strong mammalian transcriptional activator consisting of the Gal4 DNA-binding domain and the VP16 activation domain (Gal4 (1–147)-VP16) [27]. Human tissue culture cells were transfected with these constructs as well as with a plasmid expressing Gal4(1–147)-VP16 alone (positive control) or with an empty vector (negative control). For control of transfection efficiency, a plasmid expressing β -galactosidase was co-transfected following standard procedures [28]. The reporter gene to

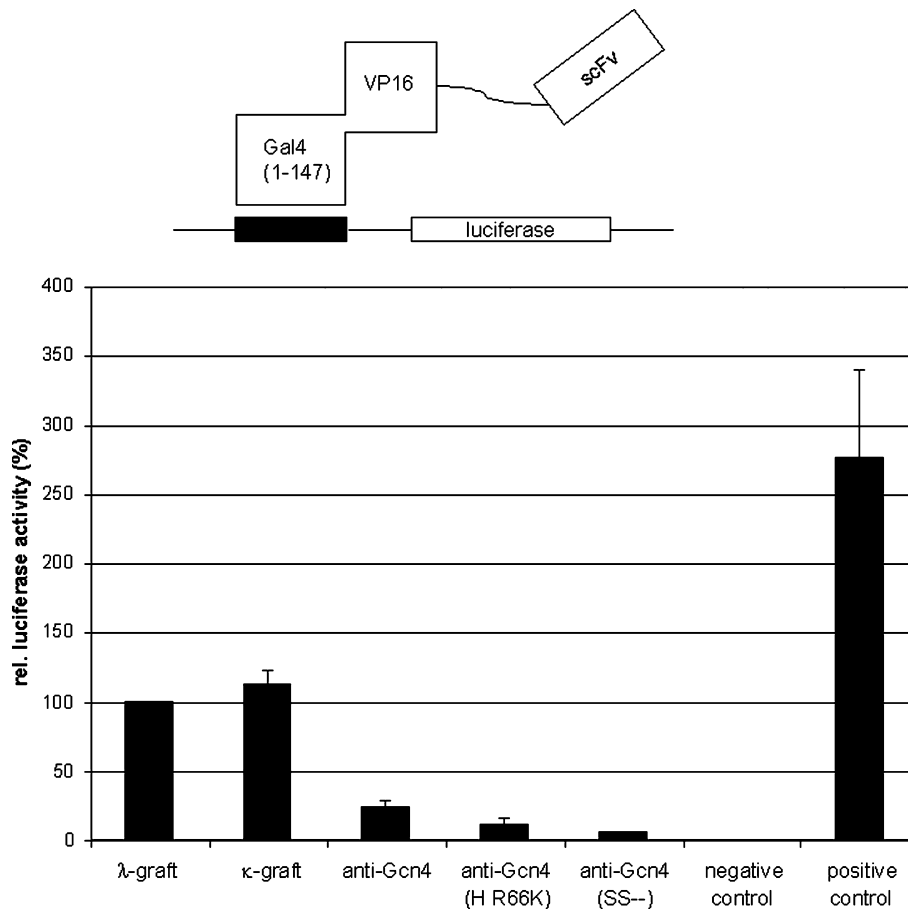


Fig. 4. Stability and solubility of various scFv fragments correlates with transcriptional activity in mammalian cells. HeGlu cells, which are HeLa cells carrying an integrated luciferase reporter gene under the control of four Gal4-binding sites, were transfected with plasmids expressing the indicated scFv variants fused to the transcriptional activator Gal4(1–147)-VP16. Gal4(1–147)-VP16 and Gal4(1–147) alone were used as positive and negative controls, respectively. Relative luciferase activity (rel. luciferase activity) elicited by the Gal4(1–147)-VP16- λ -graft fusion protein was arbitrarily set to 100%.

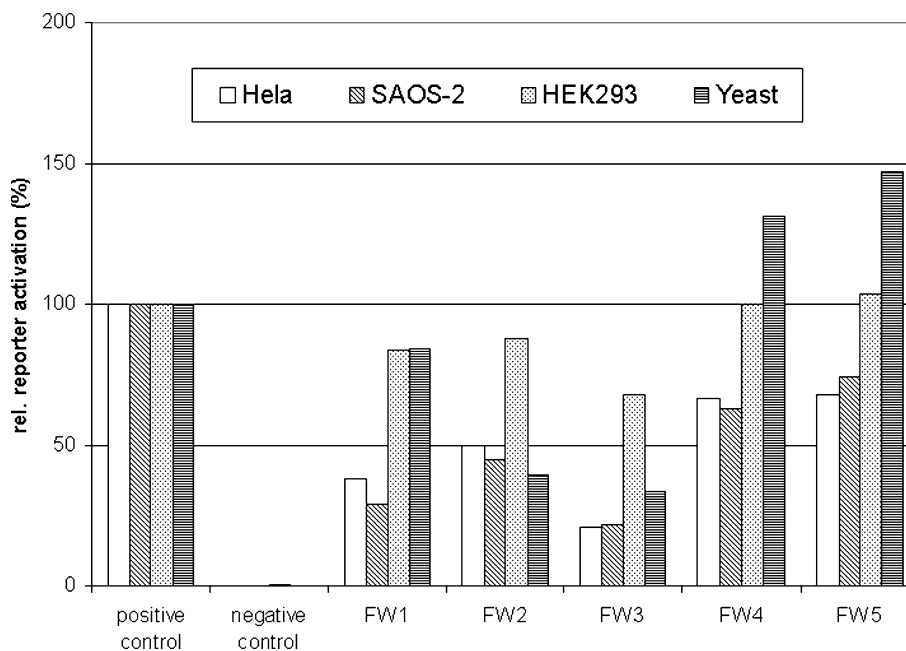


Fig. 5. The relative levels of activation of the luciferase reporter gene by the different scFv fusion proteins correlated with the levels of β -galactosidase activities elicited by the respective scFv fusion proteins in yeast. HeLa cells, SAOS-2 cells, and HEK293 cells were transfected with plasmids expressing selected scFv variants fused to the transcriptional activator Gal4(1–147)-VP16. The Gal4(1–147)-VP16- λ -graft fusion protein was used as positive control and its relative reporter activation (luciferase activity) was arbitrarily set to 100%. Gal4(1–147) was used as negative control. The same antibody frameworks were expressed in the yeast system and β -galactosidase activity was measured. Again, the LexA–Gal4AD- λ -graft fusion protein was used as positive control and the elicited reporter gene activation was arbitrarily set to 100%.

measure transcription activation, which consisted of the luciferase gene under the control of four Gal4-binding sites, was also co-transfected, except in the so-called HeGlu cells (see Fig. 4) that carry this construct integrated in their genome [29]. Cells were cultured in DMEM supplemented with 2.5% FCS and 2 mM L-glutamine. Transient transfections were carried out according to the Polyfect-protocol (Qiagen) in 60 mm tissue culture plates. A *lacZ* expression vector was used in all transfections as reference for transfection efficiency. Cells were harvested 24–48 h after transfection, resuspended in 1000 μ l buffer, and lysed by three freezing–thawing cycles. The cell lysate was centrifuged and the supernatant was assayed for luciferase activity using luciferase assay solution (Promega) and for β -galactosidase activity according to the standard protocol [28]. The obtained luciferase activity was corrected with the β -galactosidase activity to account for the variation in transfection efficiency.

We first analyzed the activity of the well-characterized anti-GCN4 scFv variants fused to the transcriptional activator Gal4(1–147)-VP16. The levels of activation of the luciferase reporter gene by the different anti-GCN4 scFv fusion proteins correlated with the levels of β -galactosidase activities elicited by the respective scFv fusion proteins in yeast (Fig. 4, compare with Fig. 2A). The λ -graft and κ -graft, which were stable and soluble in yeast, and thus allowed gene activation by the respective fusion proteins, also mediated strong activation of the

reporter gene in mammalian cells (Fig. 4). The other scFv framework variants, which did not allow reporter gene activation in yeast, could also not significantly stimulate reporter gene expression in mammalian cells (compare Fig. 4 with Fig. 2A).

A direct comparison of the performance in yeast and in mammalian cells of some frameworks selected by the “Quality Control” screening of the scFv fusion libraries is shown in Fig. 5. The relative levels of reporter gene activation in yeast cells were compared to the levels of luciferase activities elicited by the respective fusion proteins in HeLa cells, human osteosarcoma cell line SAOS-2, and the human embryonal kidney cell line HEK293. The λ -graft framework fusion protein (see above) and empty vectors were used as positive and negative controls, respectively. All scFvs, which were selected in yeast from a naïve human framework library, also allowed gene activation in all mammalian cells tested (Fig. 5). Thus, our “Quality Control” system allows exploiting the technical advantages of the yeast cells to identify intrabody frameworks that are also functional in mammalian cells.

3. Concluding remarks

Highly stable and soluble antibody fragments, such as scFv and Fab fragments, are very much desirable not only for intracellular use (i.e., as intrabodies in

functional genomics or gene therapy) but also for classical therapeutic applications towards extracellular targets and as diagnostic tools [30,31]. High stability and solubility of antibody fragments, taken together with higher tissue penetration properties due their smaller size than full-length antibodies, might afford them a unique advantage for topical applications to treat diseases that do not require systemic delivery and Fc function. Moreover, high stability and solubility of these proteins greatly facilitate their expression and purification from microorganisms such as bacteria and yeast, thus lowering costs and reducing time for production of high quality antibody fragment [32]. We have developed a system called “Quality Control” that enables simple and rapid high-throughput screen of libraries of antibody fragments to identify those that are highly stable and soluble. Our approach can also be used in a so-called “framework evolution process” (mutagenesis or sequence grafting followed by selection) to improve the quality of defined and specific antigen-binding antibodies that are poorly stable or prone to aggregation [33].

In the system described here, selection of antibody fragments endowed with high stability and solubility occurs in the intracellular (reducing) environment of yeast cells. Indeed, since the reducing environment of the cytoplasm prevents the formation of the highly conserved intradomain disulfide bridges, very stable antibody fragment frameworks can be selected which fold in the absence of the disulfide bonds and do not undergo aggregation. A peculiarity of our system is that it enables screening, identification, and analysis of stable and soluble antibody fragments independently of their antigen-binding specificities. The method has been established on the conceptual basis that stability and solubility (and therefore the activity) of a fusion protein composed of a constant, selectable marker fused to an antibody fragment is determined by the stability and solubility of the antibody moiety. We have constructed fusion proteins composed of scFv sequences fused to constant marker proteins that provide a selectable activity in yeast by controlling expression of defined reporter genes. Our results indicate that the scFv portion determines the overall stability and the function of the fusion protein. We show that the degree of reporter gene activation is directly proportional to the stability and solubility of single-chain antibody fragments that have been characterized *in vitro* for their biophysical properties. These results provide further support to the notion of a correlation between general stability and intracellular performance of antibody fragments [12]. Thus, our “Quality Control” system is suitable to select antibody fragments that are highly stable and soluble because these properties allow them to cope with the harsh intracellular conditions. With the possibility to perform growth selection assays, our system allows to rapidly and efficiently screen single-chain antibody libraries to

isolate highly stable and soluble frameworks. Frameworks selected in the “Quality Control” system subsequently serve as acceptor backbones to construct CDR libraries by randomization of one or more hypervariable loops. Such antibody libraries based on “super-stable” frameworks can be screened to identify antigen-specific binders either in a phage display format or again in yeast in a modified two-hybrid setting, in which purification of the antigen is not required [21].

Acknowledgments

We are grateful to Andreas Plückthun, Arne Wörn, and Christian Zahnd for the fruitful collaboration and for providing materials that were essential for the development of the method described here. Part of this work was supported by the Commission of Technology and Innovation (CTI) of the Swiss Government.

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